Mutated OKT3 Antibody

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The present invention relates to an H100A position point-mutated OKT3 antibody, a method for the production thereof and its use.

INS D2

OKT3 is a monoclonal IgG 2a-type antibody originating from mice, which recognizes an epitope of an ε-subunit of the human CD3 complex (Kung et al., Science 206, pp. 347-349 (1979); Van Wauwe et al., J. Immunol. 124, pp. 2708-2713 (1980); Transy et al., Eur. J. Immunol. 19, pp. 947-950 (1989)). The method of obtaining the monoclonal antibody from the corresponding hybridoma is described in detail in these publications. Furthermore, the OKT3-producing hybridoma cell line was deposited by the owner of European patent 0 018 795 under ATCC No. CRL 8001 with the American Type Culture Collection, ~~12301 Parklawn Drive, Rockville, MD, 20852~~ 10801 University Boulevard, Manassas, VA 20110-2209, on April 26, 1979. OKT3 has been used for a long time to suppress a T-cell response thus preventing the rejection of transplants (Thistlethwaite et al., Transplantation 38, pp. 695-701 (1984); Woodle et al., Transplantation 51, pp. 1207-1212 (1991)). On the other hand, OKT3 can also trigger T-cell activation and proliferation, which stimulates the effector cells, which can be used for the adoptive cancer immunotherapy (Yannelly et al., J. Immunol. Meth. 1, pp. 91-100 (1990)). OKT3 was used as such and as a component of a bispecific antibody to direct cytotoxic T-lymphocytes against tumor cells or virus-infected cells (Nitta et al., Lancet 335, pp. 368-376 (1990); Sanna et al., Bio/Technology 13, pp. 1221-1224 (1995)). Furthermore, humanized versions of the OKT3-monoclonal antibody which were expressed in COS cells are also known (Woodle et al., J. Immunol. 148, pp. 2756-2763 (1992); Adair et al., Human. Antibod. Hybridomas, pp. 41-47 (1994)). So far there has been the problem that OKT3 has no

sufficient stability and particularly cannot be expressed in known recombinant expression systems in stably fashion and sufficient amount.

Therefore, the object of the present invention was to express OKT3 recombinantly and obtain an antibody which has satisfactory stability.

This object is achieved by the subject matters defined in the claims.

The inventors have found that by introducing a point mutation at position H100A of the amino acid sequence of OKT3 the stability increases many times over. This point mutation relates to the exchange of cysteine for another polar amino acid, preferably serine, in the amino acid sequence of OKT3.

~~For the~~ production of an antibody according to the invention, mRNA from freshly subcloned hybridoma cells of OKT3 is used as a basis. The cDNA is produced according to methods known to a person skilled in the art, which were described in Dübel et al., J. Immunol. Methods 175, pp. 89-95 (1994), for example. The DNA coding for the variable domain of the light chain can be produced by means of PCR using suitable primers, e.g. by means of primers Bi5 and Bi8 which hybridize to the amino-terminal part of the constant domain of the κ-chain and the framework 1 (FR1) region of the variable domain of the κ-chain (Dübel et al., see above). For the amplification of the DNA which codes for the variable domain of the heavy chain, it is possible to use e.g. the primer Bi4 which hybridizes to the amino-terminal part of the constant domain 1 of the γ-chain (Dübel et al., cf. above) and the primer Bi3f which hybridizes to the FR1 region of the heavy chain (Gotter et al., Tumor Targeting 1, pp. 107-114 (1995).

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Thereafter, the amplified DNA is inserted in a vector adapted for sequencing and for site specific mutagenesis, as well known to the person skilled in the art. For example, the vector pCR-Skript SK(+) sold by the company of Stratagene can be used. Mutations are inserted in the V_H domain originating from OKT3 by site specific mutagenesis. The person skilled in the art is familiar with the conditions necessary for this purpose, they are also described e.g. in Kunkel et al., Meth. Enzymol. 154, pp. 367-382 (1987). The amino acid substitution at the H100A position of OKT3 (exchange of cysteine) is suitably carried out by using the primer SK1 5'-GTAGTCAAGGCTGTAATGATCATC (SEQ ID NO:7) if an exchange for serine shall be carried out at this position.

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Then, the thus modified DNA can be cloned into a vector and expression vector, respectively. The person skilled in the art is familiar with examples thereof. In the case of an expression vector, these are pGEMEX, pUC derivatives or pET3b. For the expression in yeast, e.g. pY100 and Ycpad1 have to be mentioned while e.g. pKCR, pEFBOS, cDM8 and pCEV4 have to be indicated for the expression in animal cells. The baculovirus expression vector pAcSGHisNT-1 is especially suitable for the expression in insect cells. The expression in *E. coli* is preferred according to the invention, for which purpose preferably the vector pHOG21 shown in figure 1 (Kipriyanov et al., J. Immunol. Methods 196, pp. 51-62 (1996) is used, in which the mutated OKT3 single chain (ScFv) gene is inserted as NcoI/BamHI DNA fragment. A single-chain antibody OKT3 mutated at position 100 A (Kabat numbering system) is expressed, which has the sequence shown in figure 2.

The person skilled in the art is familiar with cells adapted to express a DNA which is present in an expression vector. Examples of such cells comprise the *E. coli* strains HB101, DH1, x1776, JM101, JM109, BI21 and SG13009, the yeast strain *Saccharomyces cerevisiae* and the animal cells 3T3, FM3A,

CHO, COS, Vero and HeLa as well as the insect cells sf9. The use of the XL1-Blue *E. coli* cells sold by the company of Stratagene is preferred.

The person skilled in the art knows in which way a DNA has to be inserted in an expression vector. He is also familiar with the fact that this DNA can be inserted in combination with a DNA coding for another protein and peptide, respectively, so that the DNA can be expressed in the form of a fusion protein, e.g. in the form of a His fusion protein. The information necessary for this purpose is included in the preferably used plasmid pHOG21. Furthermore, the mutated form of OKT3 can be present in the form of a bispecific antibody, e.g. in combination with an antibody against human CD19 complex. The sequence of such a bispecific antibody is shown in figure 3.

Antibodies according to the invention distinguish themselves in that they can be produced by means of recombinant methods in sufficient amount and have a stability greater as compared to the non-mutated monoclonal antibody OKT3. This stability expresses itself e.g. in that the mutated antibody has lost almost nothing of its original binding affinity even after one month of storage at 4°C in PBS, whereas OKT3 has markedly lost binding affinity under these conditions (46 %). In addition, the antibody according to the invention has the advantage that as a single-chain antibody (scFv) it has faster blood clearance and better tumor penetration. Furthermore, ScFvs are very useful molecules to transport pharmacons, toxins or radionuclides to tumor sites, which is important for tumor diagnosis and tumor treatment.

The present invention is further described by means of the figures.

INS D5

Figure 1: plasmid pHOG21
the abbreviations used therein having the following meanings:
Ap^R: ampicillin resistance gene

c-myc: sequence coding for an epitope which is recognized by the monoclonal antibody 9E10 (Cambridge Research Biochemicals, Cambridge, Great Britain)

ColE1: origin of DNA replication

f1 IG: intergenic region of the f1 phage

His_6: sequence coding for 6 histidine residues

linker: sequence coding for 17 amino acids which links the V_H and V_L domains

pelB: signal peptide sequence for bacterial pectate lyase

P/O: wild type lac promoter / operator

Figure 2: Nucleotide sequence and derived amino acid sequence of the mutated OKT3 single-chain antibody

Figure 3: bispecific antibody composed of mutated OKT3 and anti-CD19

The invention is explained in more detail by the example.

EXAMPLE 1: Preparation of an antibody according to the invention

The isolation of mRNA from freshly subcloned hybridoma cells of OKT3 and the cDNA synthesis were carried out as described in "Dübel et al., J. Immunol. Methods 175, pp. 89-95 (1994)". The DNA coding for the variable domain of the light chain was produced by means of PCR using the primers Bi5 and Bi8 which hybridize to the amino-terminal part of the constant domain of the κ-chain and the framework 1 (FR1) region of the variable domain of the κ-chain (Dübel et al., cf. above). The primer Bi4, which hybridizes to the amino-terminal part of the constant domain 1 of the γ-chain (Dübel et al., cf. above), and the primer Bi3f, which hybridizes to the FR1 region of the heavy chain (Gotter et al., Tumor

Targeting 1, pp. 107-114 (1995), were used for the amplification of the DNA which codes for the variable domain of the heavy chain. The 50 µl reaction mixture contained 10 pmol of each primer and 50 ng hybridoma cDNA, 100 µM of each of the dNTPs, 1 x vent buffer (Boehringer Mannheim), 5 µg BSA and 1 U Vent DNA polymerase. 30 cycles were carried out per 1 minute at 95°, 1 min. at 55°C and 2 minutes at 75°C in a PCR thermocycler. The amplified DNA was purified with a QIA quick PCR purification kit (Qiagen, Hilden).

Thereafter, the amplified DNA was 'blunt-end' ligated into the vector pCR-Skript SK(+) sold by the company of Stratagene, which had been cleaved using the SrfI restriction enzyme. Mutations were inserted in the V_H domain originating from OKT3 by site specific mutagenesis (Kunkel et al., Meth. Enzymol. 154, pp. 367-382 (1987)). The amino acid substitution at position H100A of OKT3 (exchange of cysteine for serine) was carried out using the primer SK1 5'-GTAGTCAAGGCTGTAATGATGATC. (SEQ ID NO:7)

For the expression of the resulting mutated DNA the vector pHOG21 shown in figure 1 (Kipriyanov et al., J. Immunol. Methods 196, pp. 51-62 (1996) was used, in which the mutated OKT3 single chain (scFv) gene is inserted as NcoI/BamHI DNA fragment. XL1-Blue *E. coli* cells (Stratagene) were transformed with this expression vector and allowed to grow in a 2xYT medium having 50 µg/ml ampicillin and 100 mM glucose ($2xYT_{GA}$) at 37°C overnight. Dilutions (1:50) of the overnight cultures in $2xYT_{GA}$ were allowed to grow at 37°C with shaking at 37°C. As soon as the cultures had reached OD_{600} = 0.8, the bacteria were pelleted by centrifugation at 1500 g and 20°C for 10 minutes and resuspended in the same volume of fresh 2xYT medium containing 50 µg/ml ampicillin and 0.4 M sucrose. IPTG was added up to a final concentration of 0.1 mM and the growth was continued at room temperature for 20 hours. The cells were collected by centrifugation at 5000 g and 4°C for 10 minutes. The supernatant of the culture was stored on ice. In order to isolate soluble periplasmic proteins, the pelleted bacteria

were taken up in ice-cold 50 mM Tris-HCl, 20 % sucrose, 1 mM EDTA, pH 8.0 (5 % of the original volume). After one hour of incubation on ice accompanied by occasional stirring, spheroplasts were centrifuged off at 30,000 g and 4°C for 30 minutes, the soluble periplasmic extract occurring as supernatant and the spheroplasts plus insoluble periplasmic material occurring as pellet. The above supernatant of the culture stored on ice and the soluble periplasmic extract were combined and clarified by an additional centrifugation (30,000 g, 4°C, 40 min.). Following filtration by glass filters having a pore size of 10 to 16 μm and then 0.2 μm, the volume became 10 times as high by concentration with Amicon YM10 membranes (Amicon company, Witten). The concentrated supernatant was clarified by centrifugation and dialyzed against 50 mM Tris-HCl, 1 M NaCl, pH 7.0 at 4°C. Immobilized metal affinity chromatography (IMAC) was charged with Ni^{2+} at 4°C using a 5 ml column of chelatizing sepharose (Pharmacia company) and equilibrated with 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (start buffer). Material adsorbed on the column was eluted using 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, pH 7.0. Having changed the buffer to 50 mM MES, pH 6.0, the protein was further purified on a mono S ion exchange column (Pharmacia). The purified scFv antibody according to the invention was dialyzed in PBS (15 mM sodium phosphate, 0.15 M NaCl, pH 7.4). For a relatively long storage, the antibody was frozen in the presence of BSA (final concentration 10 mg/ml) and stored at -80°C.